Ultrastructure of the ascospores of some species of the Torulaspora group

Kreger-van Rij, N.J.W.; Veenhuis, M.

Published in:
Antonie van Leeuwenhoek

DOI:
10.1007/bf00410175

Publication date:
1976

Ultrastructure of the ascospores of some species of the *Torulaspora* group

N. J. W. KREGER-VAN RIJ AND M. VEENHUIS

Laboratory of Medical Microbiology and Laboratory of Electron Microscopy, Department of Biology, State University, Groningen, The Netherlands

KREGER- VAN RIJ, N. J. W. and VEENHUIS, M. 1976. Ultrastructure of the ascospores of some species of the *Torulaspora* group. Antonie van Leeuwenhoek **42:** 445–455.

Development and germination of the ascospores in species of the *Torulaspora* group of yeasts have been described. Most species had warty spores which, in sections, showed a dark outer layer consisting of the outer unit membrane of the prospore wall and a layer underneath formed at an early stage of development of the spores. In mature spores the light inner layer of the wall was delimited at the outside by a thin dark layer. The warts often contained dark material. The ascospores of two *Pichia* and three *Debaryomyces* species were studied for comparison; they differed in sections from the *Torulaspora* spores. The taxonomic implications of the ultrastructural observations have been discussed.

INTRODUCTION

The genus *Torulaspora* in the descriptions of Lindner (1904), Guilliermond (1928) and Stelling-Dekker (1931) includes haploid yeasts with spherical cells producing conjugation tubes and smooth spherical ascospores. Nitrate assimilation was absent and sugar fermentation was strong. Lodder and Kreger-van Rij (1952) did not accept the genus *Torulaspora* and transferred the species to the genus *Saccharomyces*: van der Walt (1970) retained this classification. Meanwhile, some of the former *Torulaspora* species were found to have warty spores (Kudrjawzew, 1960; Kodama et al., 1964; Kreger-van Rij, 1966; Kreger-van Rij, 1970; Kurtzman, Smiley and Baker, 1975), a property also typical of the genus *Debaryomyces*.

According to Kreger-van Rij (1970), it was possible to recognize a group *Torulaspora* in *Saccharomyces* characterized as follows: "cells spherical, no pseudomycelium; formation of protuberances which may act as conjugation tubes; vigorous fermentation of sugars; of the polyalcohols only mannitol and

glucitol may be assimilated; no assimilation of β-glucosides and pentoses; growth occurs without the addition of vitamins". Moreover, this author found a difference in the structure of the warty spore wall in ultra-thin sections between *Torulaspora* and *Debaryomyces* which required, however, further examination.

Recently, van der Walt and Johannsen (1975) have re-established the genus *Torulaspora* including in it haploid, budding, nitrate-negative yeasts with spherical or oblate-ellipsoidal spores without a ledge. The definition comprises, apart from the *Torulaspora* group, species of the genus *Debaryomyces* (sensu Lodder et Kreger-van Rij) and several *Pichia* and *Saccharomyces* species.

We think that by this definition the structure of the spore wall is insufficiently considered. Since knowledge about it is incomplete, we have studied the ultrastructure of ascospores in some species of the *Torulaspora* group. The spores of some *Pichia* and *Debaryomces* species now described as *Torulaspora* species by van der Walt and Johannsen, were examined for comparison.

MATERIALS AND METHODS

The following strains of species of the *Torulaspora* group have been examined: *Saccharomyces kloeckerianus* van der Walt (= *Debaryomyces globosus* Klöcker), CBS 5500 and 5503; *Saccharomyces fermentati* (Saito) Lodder et Kreger-van Rij, G 62; *Saccharomyces rosei* (Guilliermond) Lodder et Kreger-van Rij, G 144 and 434; *Saccharomyces delbrueckii* Lindner, CBS 4804; *Saccharomyces pretoriensis* van der Walt et Tscheuschner, CBS 2187; *Saccharomyces inconspicuus* van der Walt, CBS 3003; *Saccharomyces vafer* van der Walt, CBS 2733; *Torulaspora nilssonii* Capriotti, CBS 2734 and 4686, and *Torulaspora franciscae* Capriotti, CBS 2926 and 5080.

Of the genera *Debaryomyces* and *Pichia* the following strains were studied: *Debaryomyces formicarius* Golubev et Bab'eva, CBS 6454; *Debaryomyces vanriji* (van der Walt et Tscheuschner) Abadie, Pignal et Jacob, G 276; *Debaryomyces yarrowii* Santa Maria et Aser, CBS 6246; *Pichia etchellsii* Kreger-van Rij, G 6, and *Pichia vini* (Zimmermann) Phaff, CBS 4050.

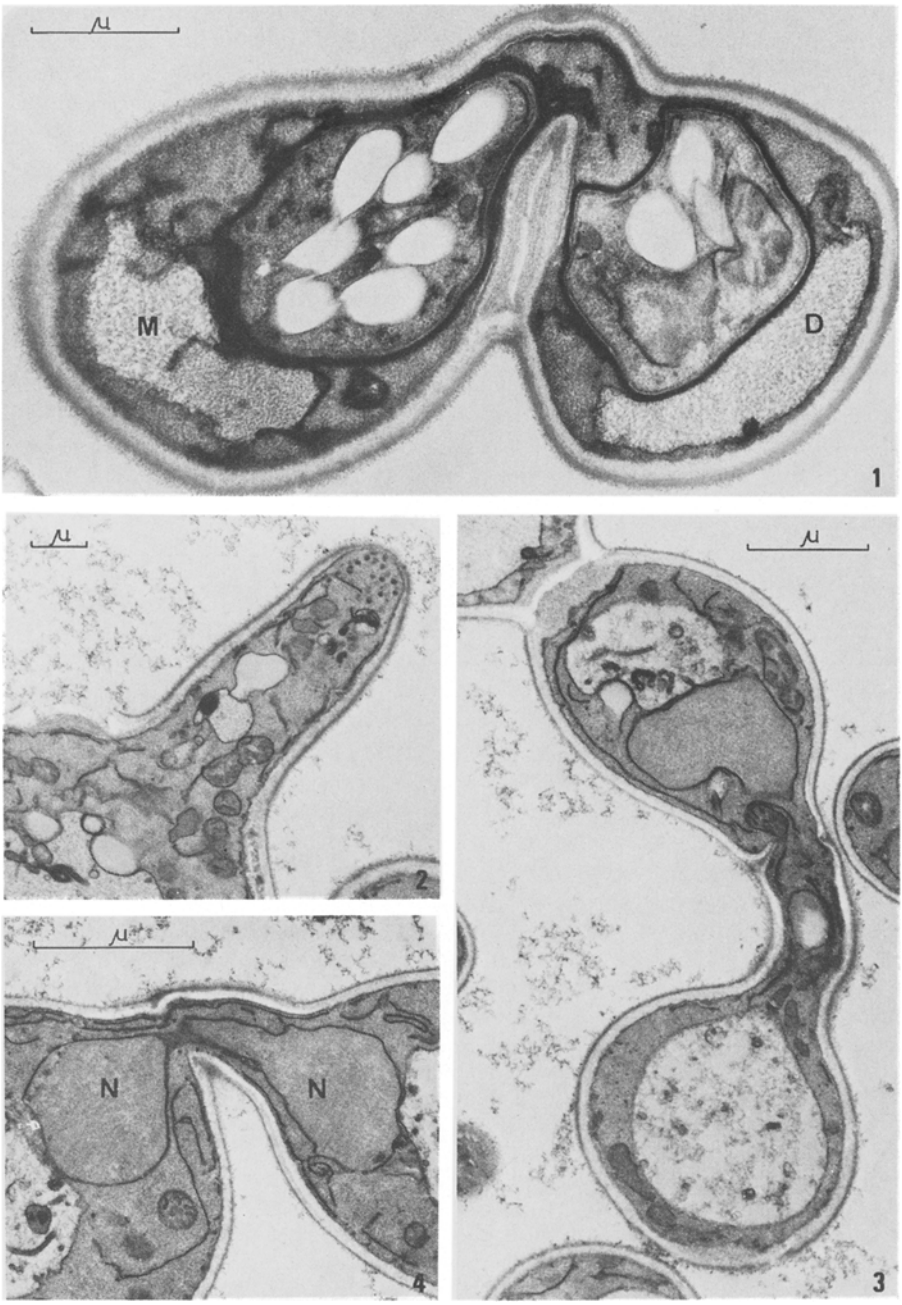
The photographs are made of sections of $KMnO_4$-fixed material, unless stated otherwise. The marker represents 0.1 μ, unless mentioned otherwise.

Fig. 1. Ascus of *Saccharomyces pretoriensis* originated from conjugation between mother cell (M) and daughter cell (D). Part of the cross wall between the cells is still present. In both cells one young spore is visible. In the wall of the spores a dense outer layer and a thin light inner layer are visible.

Fig. 2. Conjugation tube on a cell of *S. kloeckerianus*. The tip of the tube contains many vesicles.

Fig. 3. Conjugation of two cells of *S. kloeckerianus;* the bottom cell has a conjugation tube, the top cell, a bud still attached to the mother cell, forms a slight protuberance.

Fig. 4. Fusion of the nuclei (N) in the open connection between two cells of *S. kloeckerianus*.


M
D
1
2
N
N
4
3

The strains were grown on various media suitable for obtaining sporulation. The two strains of *S. kloeckerianus* were mixed in a thick suspension and the mixture streaked out on 5% malt extract agar. After 5 days at 25 C, sporulation was abundant. A preparation of germinating spores of the strain of *S. fermentati* was obtained from a 16 h slide culture with glucose–yeast extract–peptone agar inoculated with a sporulating culture.

Some of the preparations were made recently, some of them 10–15 years ago. The latter had been fixed with 1% w/v OsO_4 in veronal–acetate buffer (pH 6.0) for 16 h at room temperature, and postfixed with 0.5% w/v aqueous uranyl acetate solution for 1 h. The material was dehydrated through an acetone series and embedded in Vestopal. The recent preparations were fixed (1) with 1.5% w/v $KMnO_4$ solution for 20 min at room temperature or (2) after fixation with 1.5% $KMnO_4$ for 20 min, with 1% OsO_4 solution for 2 h at room temperature. The fixed material was washed, dehydrated with ethanol and during dehydration poststained with a saturated solution of uranyl acetate in 100% ethanol for 1 h. The cells were embedded in Epon or Spurr's resin (1969). Sections were sometimes stained with lead citrate.

RESULTS

In the species of the *Torulaspora* group conjugation preceding ascus formation occurred by various methods: (1) between mother cell and daughter cell (Fig. 1). Very often, the daughter cell was one of many small buds still attached to the mother cell but separated from it by a cross wall (so-called Kronenbildung). (2) between two cells with conjugation tubes. These (Fig. 2) fused at the tips. (3) between a cell with a conjugation tube and one without (Fig. 3). The latter cell formed a slight protuberance, possibly in response to contact

---

Fig. 5. Prospore wall (arrow) lying around part of a nucleus (N).

Fig. 6. Part of the prospore wall delimited by two unit membranes (arrows) in a cell of *S. kloeckerianus*. The membrane of a vacuole (V) is also visible.

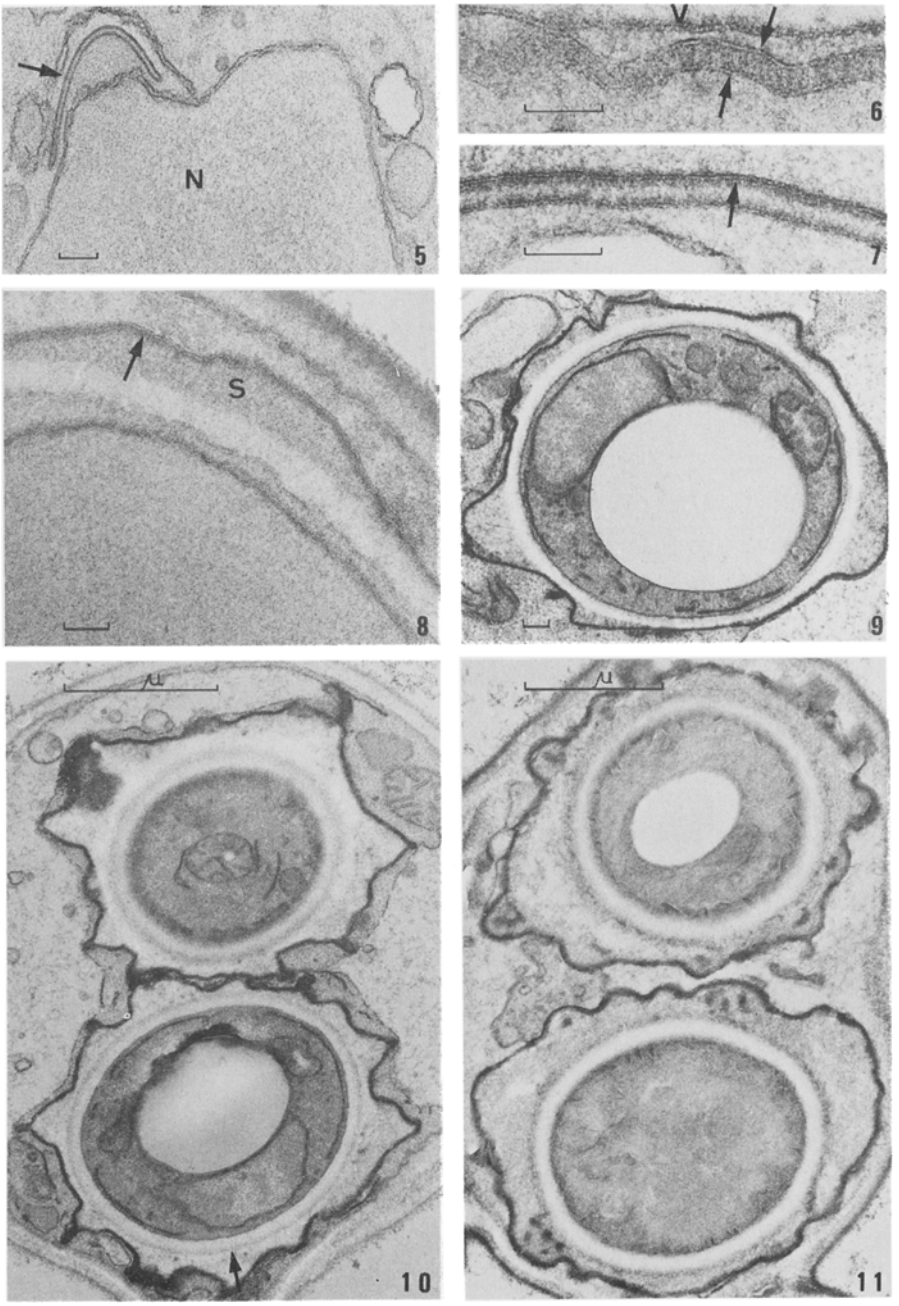
Fig. 7. Prospore wall of *S. kloeckerianus* with a thin dense layer lying close to the outer unit membrane (arrow).

Fig. 8. Part of a section through a young spore of *S. rosei* with a swelling (S) which is the initiation of a wart. Under the outer unit membrane of the wall a dark layer is visible (arrow). The swelling is greyish and the inner layer of the wall is electron-light.

Fig. 9. A young spore of *S. kloeckerianus* with distinct, grey warts.

Fig. 10. Two spores of *S. kloeckerianus*. The warts are pointed. The outer layer of the spore is very dark. The inner layer is light with a thin dark line around it (arrow). In the top spore, the wall outside the dark line has partly extended; the warts are still greyish.

Fig. 11. Mature spores of *S. rosei*. The dark outer layer and light inner layer of the wall are distinctly visible. In both spores the wall outside the dark line around the light inner layer has extended. In the warts some dark material is visible.


N
5
V
6
7
S
8
9
10
11

with the conjugation tube. The three types of conjugation were observed in all species of the *Torulaspora* group, often in the same strain. Of the two strains of *S. kloeckerianus* examined, one formed conjugation tubes and the other showed "Kronenbildung" and produced no conjugation tubes on sporulation media. The strains behaved as mating types in that they did not sporulate alone, or very sparsely, but conjugated and sporulated readily when mixed. Asci with protuberances and without evidence of conjugation have been described for all species of the *Torulaspora* group; we found a few of them in most strains studied.

We observed stages of nuclear fusion in sections of conjugating cells (Fig. 4). One to four spores were formed in each ascus, either in one of the fused cells or, more frequently, distributed over both. In the latter case, it seems likely that meiosis took place at the site of fusion. In older cultures of *S. fermentati* free spores were found.

We studied development of the ascospores in strains of the species *S. kloeckerianus*, *S. fermentati* and *S. pretoriensis*, and examined mature ascospores of *S. rosei*, *S. delbrueckii*, *S. inconspicuus*, *S. vafer*, *Torulaspora francisae* and *T. nilssonii*.

In sections, the first stage of spore formation observed was the prospore wall consisting of two unit membranes lying close together (Figs. 5, 6).

Initially, the space between the membranes was often electron-dense; it differentiated into a thin dense layer under the outer membrane (Fig. 7) and a lighter layer. The dark layer persisted during maturation; it was of even thickness and density and, especially in mature spores, sharply outlined (Fig. 13). In part of the OsO_4-fixed material, it was less dark than after fixation with $KMnO_4$, but still distinctly visible (Fig. 12). Stages in the development of the spores after the formation of the prospore wall showed a still thin wall with slight swellings as the beginning of warts (Fig. 8). These became more distinct protuberances in mature spores, rather pointed in *S. kloeckerianus* (Fig. 10) and rounded in *S. rosei* (Fig. 11). The wall of the young spore was, apart from the dark outer layers greyish with a light inner layer. While the latter became wider, the greyish material was nearly completely confined to the warts (Fig. 9). During the last stages of maturation the inner layer turned lighter and was delimited by a distinct thin dark layer from the outer part of the wall (Figs. 10, 11). The spore wall at the outside of this dark layer extended and became lighter, occasionally with some darker material left in the warts which partly changed in shape (Figs. 10, 11, 13). We observed this extension of the spore wall in all strains studied, but it is not excluded that it is an artefact caused by the preparation of the cells.

During germination of the spores of *S. fermentati*, the protoplast swelled with a new light layer surrounding it. The warts were flattened, but the dark outer layer was still visible (Figs. 14, 15). It was often broken up and, occasionally, loosened from the germinated spore.

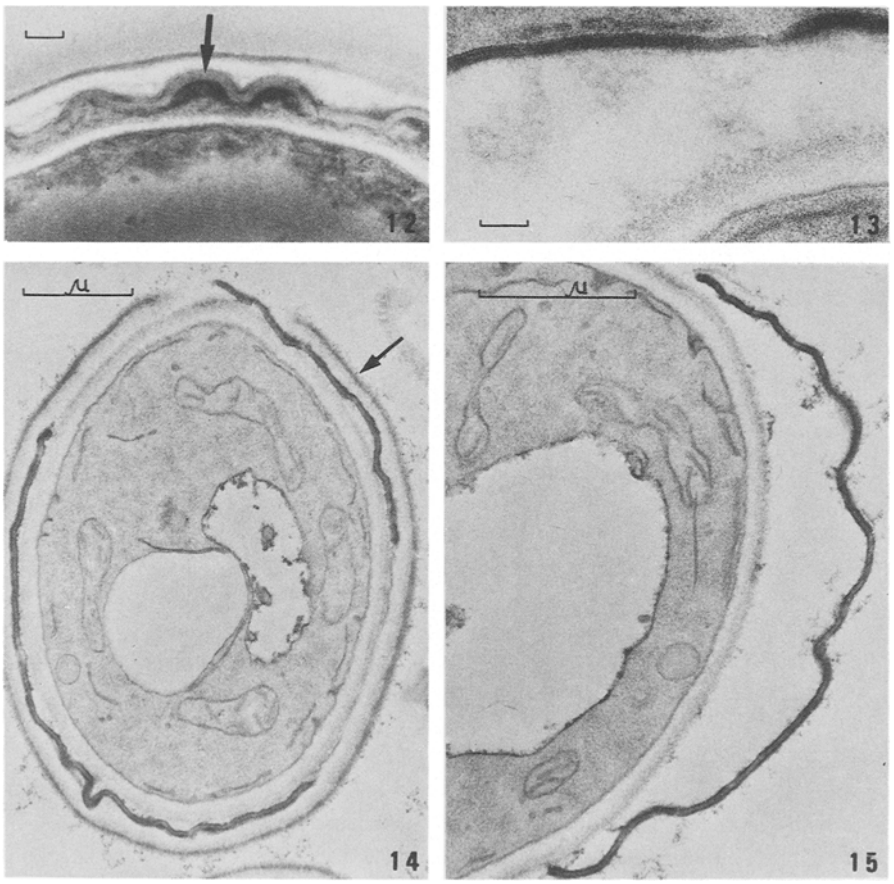


Fig. 12. Section through a spore in an ascus of *S. rosei* fixed with OsO_4. The top dark and light layers constitute the ascus wall. The wavy layer of even thickness under the thin dark outer layer of the spore wall is distinctly visible (arrow). The warts partly contain dark material.
Fig. 13. Part of the wall of a mature spore of *S. fermentati* with an extended wall. The warts have disappeared completely. The dark layer under the outer membrane is distinct. The inner layer is light-greyish with a darker part delimiting it from the rest of the wall which is light with grey spots.
Fig. 14. Germinated spore of *S. fermentati*. The protoplast has swollen and, with the layers around it, fills the ascus (arrow). The dark outer layer of the spore wall is broken.
Fig. 15. Germinated free spore with a loosened dark layer.

The spores of the type strain of *S. pretoriensis* were smooth. Their wall also developed from a prospore wall with an electron-dense layer under the outer membrane (Fig. 1). The warts were lacking, but a thin layer of dark material was sometimes found under the dark outer layers.

The spores of *Debaryomyces yarrowii*, *D. formicarius* (Fig. 16) and *D. vanriji* had warts which, in sections, were dark grey, sometimes with darker spots. The broad inner layer was light and the outer layer, covering the warts, was thin and dark; occasionally it could be seen that it consisted of two dark lines. Very young spores had a wall with a greyish inner layer including the warts which were only slight swellings, and a thin dark outer layer originating from the outer unit membrane (Fig. 17).

The wall of mature spores of *Pichia etchellsii* and *P. vini* consisted of a broad electron-light inner layer and a thin electron-dense outer layer in which the two dark lines of the outer membrane of the prospore wall could still be recognized (Fig. 19). The surface of the wall was occasionally slightly dented. In very young spores the outer membrane was already darker than the inner one (Fig. 18).

DISCUSSION

The spores of the species in the *Torulaspora* group studied are, with one exception, uniform in ultra-thin sections, with as conspicuous properties: warts, a typical, more or less electron-dense layer under the thin outer layer, and a very light inner layer delimited by a thin dark layer. The spores of *S. pretoriensis* have no warts.

The electron-light inner layer of the wall is found in all kinds of yeast ascospores. The delimitation of the light layer from the rest of the spore wall by darker material is not unusual either, but the thin dark layer is very conspicuous in the *Torulaspora* group.

A comparison of the *Torulaspora* spores with those of the *Debaryomyces* and *Pichia* species studied shows that the dark layer under the outer unit membrane of the prospore wall in *Torulaspora* is absent in *Debaryomyces* and *Pichia*. The *Debaryomces* species agree with most *Torulaspora* species in the wartiness of the spore wall, but *P. etchellsii* and *P. vini* lack warts. Moreover, the species of the *Torulaspora* group differ from the *Debaryomyces* and *Pichia* species in the rate of fermentation and in assimilation reactions. We think that the structure of the spore wall combined with the physiological properties suffice to keep the *Torulaspora* species and the *Debaryomces* and *Pichia* species in separate genera, and we disagree with the proposal of van der Walt and Johannsen (1975) to include them in the genus *Torulaspora*.

In the genus *Saccharomyces* (sensu Lodder et Kreger- van Rij) a group of haploid species, formerly classified in the genus *Zygosaccharomyces*, closely resembles the species of the *Torulaspora* group. Van der Walt and Johannsen

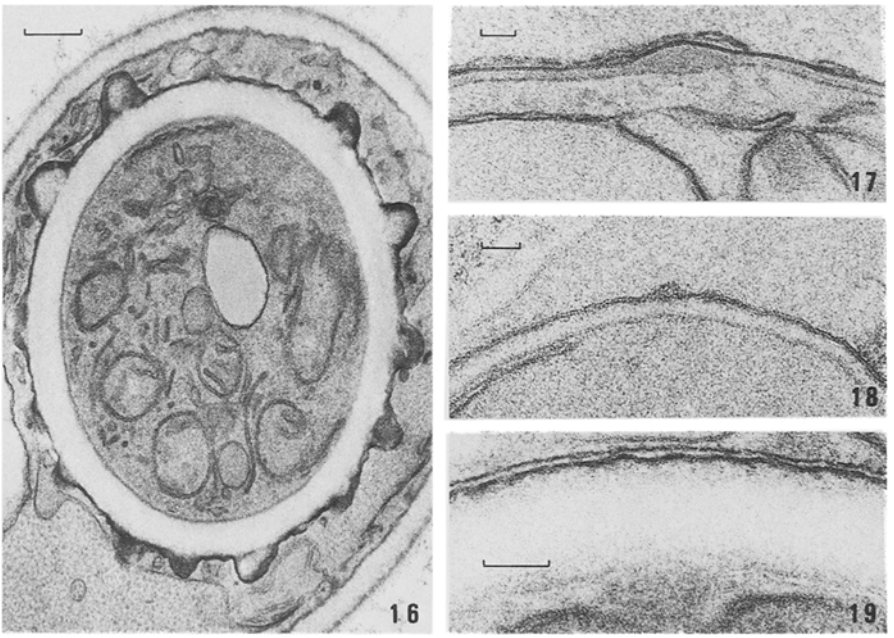


Fig. 16. Section through an ascospore of *Debaryomyces formicarius*. The spore wall is warty and it has a light inner layer; the warts are greyish with dark spots.
Fig. 17. Part of the wall of a young spore of *D. formicarius* showing a developing wart as a slight swelling. The wall is grey and has a dark outer layer originating from the outer unit membrane of the prospore wall.
Fig. 18. Section through a young spore of *Pichia vini*. The outer membrane of the prospore wall is darker than the inner one, the plasmalemma.
Fig. 19. Fragment of the wall of a mature spore of *P. vini* with a thin dark outer layer and a broad light inner layer.

(1975) have transferred part of the *Zygosaccharomyces* species to the genus *Torulaspora* while retaining the others in a re-established genus *Zygosaccharomyces*. However, the diagnosis of *Torulaspora* (including *Debaryomyces* and *Pichia* species) and the characteristics of *Zygosaccharomyces* mentioned by these authors do not show a sharp distinction between the two genera, although some differences are indicated. In the first place, in *Zygosaccharomyces* diploidization occurs as a rule by fusion of independent cells and in *Torulaspora* by "somatogamous autogamy", but both types of conjugation are found in both groups. Van der Walt (1970) uses the term autogamy, i.e. "karyogamy in the absence of cellular fusion" (Hartmann, 1929) incorrectly for conjugation between mother cell and daughter cell. Kreger-van Rij and Veenhuis (1975) have shown for the species *Debaryomyces hansenii* that in conjugation between mother and daughter cell, the fusing cells are first separated by a complete

wall. The same holds for conjugation between mother cell and bud in *Torulaspora* and *Zygosaccharomyces*. Fusion of the cells seems to be preceded by outgrowths of the cells at the cross wall. In this respect it may be similar to conjugation between different cells with conjugation tubes. Conjugation between mother cell and bud is often found in cells forming many small buds (Kronenbildung). Cells with "Kronenbildung" and cells forming conjugation tubes are separately found in the two so-called mating type strains of *S. kloeckerianus*. It is still a question whether these represent true mating types, and the mechanism and conditions of the two types of fusion mentioned above have yet to be examined. It is not known how diploidization occurs in asci of *Torulaspora* and *Zygosaccharomyces* species with a conjugation tube, but without evidence of conjugation.

A second difference between *Torulaspora* and *Zygosaccharomyces* mentioned by van der Walt and Johannsen (1975) is in the outside structure of the spore wall: "usually warty in *Torulaspora* and smooth in *Zygosaccharomyces*". Since *Torulaspora* includes smooth-spored species as *S. pretoriensis*, this difference is not decisive either. Here also, the internal structure of the ascospores may bring about additional characteristics.

Summarizing, we think that re-establishment of the genus *Torulaspora* is premature, and the inclusion of *Debaryomyces* and *Pichia* species in it is not warranted.

This investigation was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). The authors are indebted to Mr. J. Zagers for help in the preparation of the photographs and to Mr. D. Yarrow for corrections of the English text.

Received 7 *August* 1976

REFERENCES

GUILLIERMOND, A. 1928. Clef dichotomique pour la détermination des levures. — Librairie le François, Paris.

HARTMANN, M. 1929. Fortpflanzung und Befruchtung als Grundlage der Vererbung. — Verlag von Gebrüder Borntraeger, Berlin.

KODAMA, K., KYONO, T., NAGANISHI, H. and TAKAHARA, Y. 1964. Taxonomic studies on the genus *Debaryomyces*. III. *Debaryomyces franciscae* (Capriotti) nov. comb. — J. Ferment. Technol. **42:** 1–6.

KREGER-VAN RIJ, N. J. W. 1966. Some features of yeast ascospores observed under the electron microscope. *In* Proc. Second Symposium on Yeasts, Bratislava.

KREGER-VAN RIJ, N. J. W. 1970. The genus *Debaryomyces* Lodder et Kreger-van Rij, p. 129–156. *In* J. Lodder, (ed.), The Yeasts. — North-Holland Publ. Co., Amsterdam.

KREGER-VAN RIJ, N. J. W. and VEENHUIS, M. 1975. Electron microscopy of ascus formation in the yeast *Debaryomyces hansenii*.— J. Gen. Microbiol. **89:** 256–264.

KUDRJAWZEW, W. I. 1960. Die Systematik der Hefen. — Akademie Verlag, Berlin.

KURTZMAN, C. P., SMILEY, M. J. and BAKER, F. L. 1975. Scanning electron microscopy of ascospores of *Debaryomyces* and *Saccharomyces*. — Mycopathologia **55:** 29–34.

LINDNER, P. 1904. Neue Erfahrungen aus dem letzten Jahre in Bezug der Hefen und Gärung. — Jahrb. Versuchs- Lehranstalt Brau. Berlin **7:** 441–464.

LODDER, J. and KREGER- VAN RIJ, N. J. W. 1952. The Yeasts, a taxonomic study. — North-Holland Publ. Co., Amsterdam.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. — J. Ultrastruct. Res. **26:** 31–43.

STELLING-DEKKER, N. M. 1931. Die sporogenen Hefen. — Verh. K. Acad. Wet. Sect. II **28:** 1–547.

VAN DER WALT, J. P. 1970. The genus *Saccharomyces* (Meyen) Reess, p. 555–718. *In* J. Lodder, (ed.), The Yeasts. — North-Holland Publ. Co., Amsterdam.

VAN DER WALT, J. P. and JOHANNSEN, E. 1975. The genus *Torulaspora* Lindner. — CSIR Research Report no. **325:** 1–23.